

**Bin 4**

**Bin 2**

**Bin 1**

**Legend**

subTOM - calls external utilities

external packages

subTOM - calls internal functions

subTOM - optional internal functions

**first stage** →

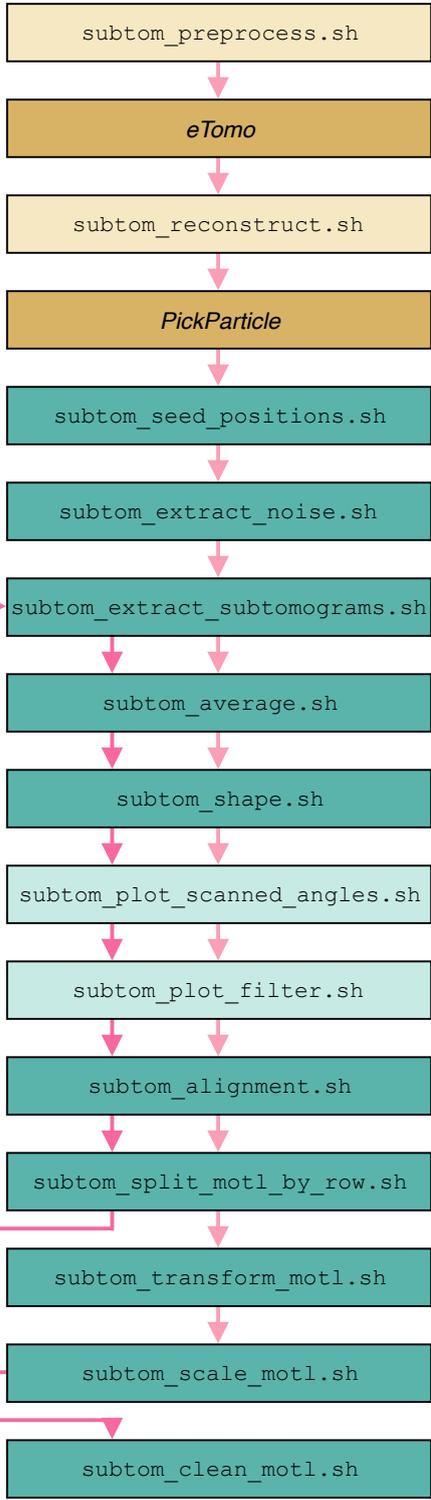
**second stage** →

**third stage** →

**fourth stage** →

**fifth stage** →

**final stage** →



## Tomogram generation

Dose-fractionated movies are aligned and converted into tilt-series, and their defocus is estimated. Tilt-series are aligned by the user and then tomograms are reconstructed in batch in NovaCTF.

## Particle Picking

Particles are picked using a UCSF Chimera plugin, assuming a geometry of points distributed normal to the surface of spheres or tubes.

## Initial Averaging

Particles are extracted from the tomogram along with a per-tomogram Fourier weight which is estimated from noise in the tomogram. The weight is used in place of the traditional binary missing-wedge to compensated for anisotropic sampling, and a weighted initial average is generated from the picked coordinates and orientations.

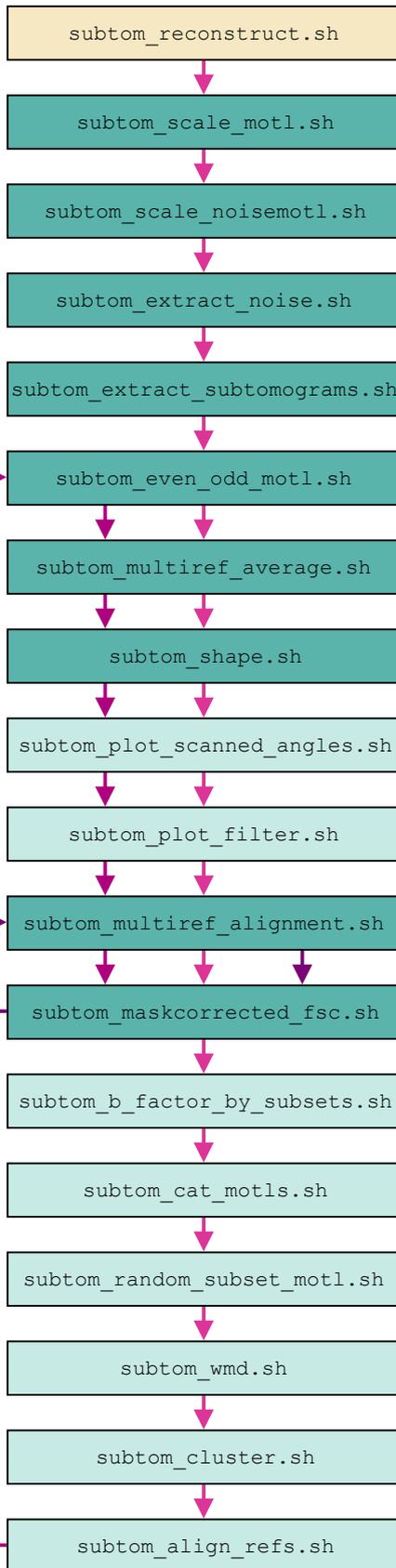
## Alignment

Masks are created to focus the correlation and constrain translations. Alignment is carried over user-specified local rotations and within a selected band-pass frequency range. The angles and filters can be visually inspected using optional plot utilities.

## Geometric Analysis

Progress of the alignment can be inspected visually using UCSF Chimera. The position of all particles can be adjusted by means of an affine transformation from the picked positions. Large scale transformations can be reset to new particle extract coordinates to recenter the particles after the transform. Finally duplicate particles and low-score particles can be removed from the dataset by cleaning.

# Tomogram generation



Processing usually begins with non-CTF corrected tomograms, but tomograms are re-reconstructed with 3D-CTF correction for higher resolutions.

## Gold-Standard Averaging

Particles and noise coordinates are first scaled from bin4 to bin2 and the shifts are reset before being extracted from the tomogram along with updated weights. The particles are then split into halves required for gold-standard refinement and a weighted average is generated for each half.

## Gold-Standard Alignment

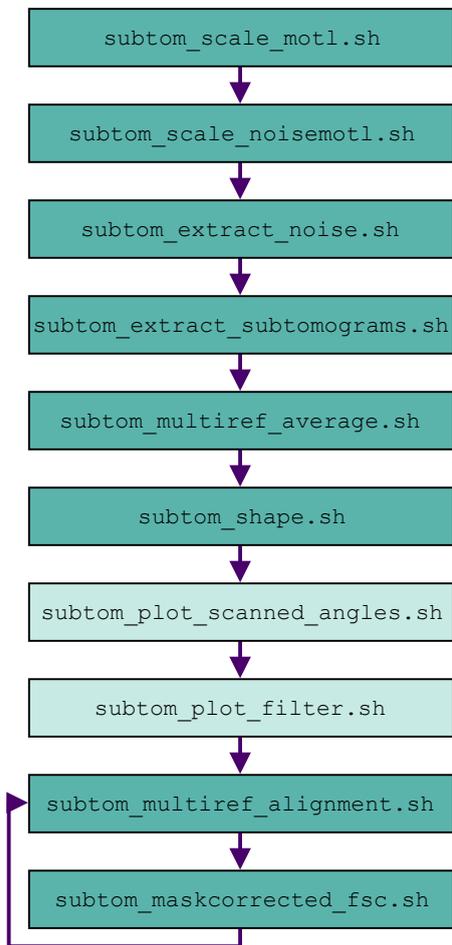
New tighter masks are created to focus the correlation and constrain translations. Alignment is now carried over user-specified local rotations and within a selected band-pass frequency range for each half-set independently.

## Resolution Analysis

Progress of the alignment can be measured by resolution determined by a mask-corrected FSC. Optionally the B-factor can be estimated by the resolution of subsets as an additional indicator.

## Classification

The halves can be concatenated so the entire dataset can be classified. Several classification methods all based on Multivariate Statistical Analysis (MSA), including the Wedge-Masked Difference method here, exist. Factors can be determined on a subset of the data, the whole dataset then projected on this basis for clustering to speed up computation. Finally class averages can be aligned to each other and propagated to all particles to continue alignment.



## Gold-Standard Averaging

Particles and noise coordinates are again scaled, now from bin2 to bin1 and the shifts are reset before being extracted from the tomogram along with updated weights and a weighted average is generated for each half.

## Gold-Standard Alignment

The final masks are created to focus the correlation and constrain translations. Alignment is again carried out over user-specified local rotations and within a selected band-pass frequency range for each half-set independently.

## Resolution Analysis

Progress of the alignment can be measured by resolution determined by a mask-corrected FSC.